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# A Novel Suppressor of Cell Death in Plants Encoded by the *Lls1* Gene of Maize

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## Summary

**The *lls1* (*lethal leaf spot1*) locus of maize is defined by a recessive mutation characterized by the initiation, in a developmentally programmed manner, of necrotic lesions that expand to kill leaves cell autonomously. The loss-of-function nature of all *lls1* mutants implies that the *Lls1* gene is required to limit the spread of cell death in mature leaves. We have cloned the *Lls1* gene by tagging with *Mutator*, a transposable element system in maize, and we show that it encodes a novel protein highly conserved in plants. Two consensus binding motifs of aromatic ring-hydroxylating dioxygenases are present in the predicted LLS1 protein, suggesting that it may function to degrade a phenolic mediator of cell death.**

## Introduction

The *lethal leaf spot* mutation of maize, which led to the discovery of the *lls1* gene, was first reported by Ullstrup and Troyer in 1967. It is inherited in a recessive monogenic fashion and is characterized by the formation of developmentally specified, randomly scattered necrotic leaf spots (lesions) that expand continuously to engulf the entire tissue. *lls1* spots show a striking resemblance to lesions incited by race 1 of Cochliobolus (Helminthosporium) carbonum, so this mutation has been grouped among a class of genetic defects in maize called "disease lesion mimics." These mutations, which cause discrete disease-like symptoms in the absence of pathogens, map to at least forty loci in maize, a majority of which exhibit dominant inheritance, making them the largest class of gain-of function mutations in maize (Walbot et al., 1983; Johal et al., 1995). Similar mutations have also been discovered in other plants including Arabidopsis and barley, and because of their pathophysiological significance, they have become the focus of intense research (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Dangl et al., 1996; Freialdenhoven et al., 1996; Jabs et al., 1996).

Little is known about the mechanistic basis of the disease lesion mimic phenomenon in plants. Based on the logic that there are two phases of lesion expression, initiation and propagation, a genetic model was proposed to explain lesion mimics (Walbot et al., 1983). This model assumes that cell damage initiated by any means is autocatalytic and will continue to expand unless checked by a suppression system. Dominant lesion mimic mutants are proposed to result from the inappropriate production of a factor(s) that mimics the cell-damaging effects of pathogen invasion, while recessive mimic mutations cause the system(s) that contains cell damage to fail.

An alternative model proposes that lesion mimics result from alterations in the disease resistance (R) genes of plants (Pryor, 1987). When plants recognize an incompatible pathogen, a hypersensitive cell death reaction (HR) is triggered in the challenged cell(s) (Staskawicz, et al., 1995; Bent, 1996). Conceivably, inappropriate activation of an R gene by a mutation could result in uncontrolled cell death and, hence, a lesion mimic phenotype (Pryor, 1987; Johal et al., 1995). Direct support for this model comes from the identification of alleles of the *Rp1* gene of maize that confer lesion mimic phenotypes (Pryor 1987; Hu et al., 1996). In addition, a number of histological, biochemical, and molecular markers normally associated with the HR are upregulated in many lesion mimic mutations (Wolter et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994), suggesting a causal link between the phenomena of disease resistance and lesion mimics.

A third model is based on the premise that lesions are caused by defects in the regulation of programmed cell death (PCD), which is either triggered precociously or not contained adequately in lesion mimic mutations (Johal et al., 1995; Dangl et al., 1996). As in animals, cell death is an integral part of both plant development and defense. In addition to the HR, examples of PCD in plants include xylem differentiation, gamete development, dissolution of endosperm or cotyledons during seed germination, leaf senescence, and flower senescence following pollination (Gahan, 1981; Johal et al., 1995; Jones and Dangl, 1996). It is likely therefore that, as in animals (Jacobson et al., 1997; Nagata, 1997), a complex molecular machinery has evolved to implement programmed cell death in plants, aberrant regulation of which will have dire consequences for the plant, including the defects that manifest as lesion mimics.

Amid a number of probable mechanisms that can cause cell death, the inappropriate production of free radicals may be the most common (Hockenbery et al., 1993; Kane et al., 1993). As in animals, most cell deaths in plants, including the HR, appear to be caused by free radicals (Hammond-Kosack and Jones, 1996). The *lsd1* (*lesions simulating disease*) mutation of Arabidopsis, one of the few lesion mimic mutations to be studied in detail, is mediated by the production of superoxide (Jabs et al., 1996). The wild-type *Lsd1* gene appears to negatively regulate cell death by inhibiting signals for superoxide production (Jabs et al., 1996).

Although it is not known yet what mechanism causes the initiation and propagation of cell death in *lls1*, the genetic and phenotypic behavior of *lls1* suggest that, like the *Lsd1* gene in Arabidopsis, the wild-type *Lls1* gene is a suppressor of cell death in maize. To gain an

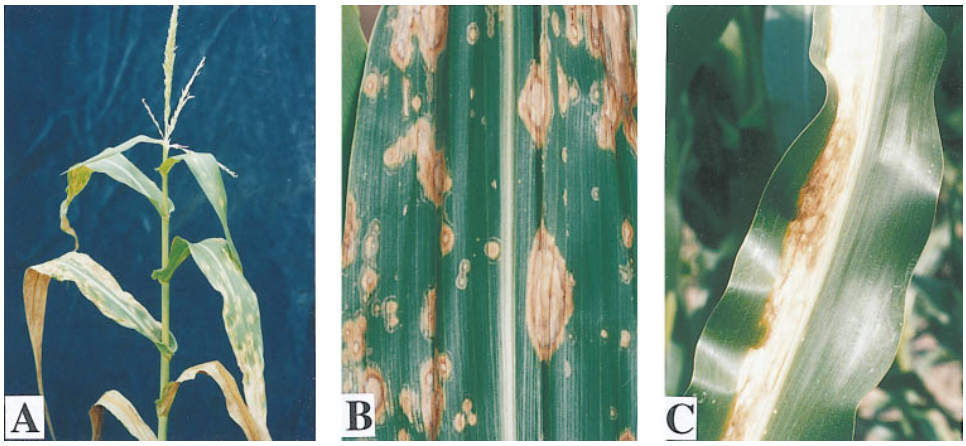


Figure 1. Phenotypic Features of the *lls1* Mutation

(A) Typical pattern of the *lls1* mutation on an 8-week-old plant.
(B) Close-up of expanding *lls1* lesions showing the presence of concentric rings.
(C) A forward-mutation somatic sector of *lls1* in an *Lls1*/*lls1-ref* plant that was observed during the directed transposon mutagenesis of *Lls1*.

insight into the molecular mechanism(s) by which *lls1* inhibits cell death, we have cloned this gene by transposon tagging. It encodes a novel protein that is highly conserved in plants. Structural features of the deduced protein suggest that the cell death–repressible activity of LLS1 may be mediated by the detoxification of a phenolic compound.

## Results

### Developmentally Programmed *lls1* Lesions Exhibit Cell Autonomy

A key feature of *lls1* mutations, irrespective of their origin or mechanism of generation, is the pattern of expression. Lesions are first observed as small chlorotic flecks near the tip of the first leaf when the seedlings are 3–4 weeks old. These lesions enlarge and coalesce, as new lesions progressively initiate down the leaf blade, following an age gradient (Figures 1A and 1B). Lesions blight the whole leaf within a few days. Meanwhile, lesions have already started near the tip of the second leaf. This pattern continues until the entire plant is blighted shortly after pollen shed. Although the leaf tissue becomes necrotic on *lls1* plants, lesions are rarely observed on other organs. Genetic mosaics of *lls1*, recovered either as forward mutations (Figure 1C) or as revertant sectors (Johal et al., 1994), indicate that the effect of the *lls1* mutation is confined to mutant cells (that is, it is cell autonomous).

### Tagging and Cloning of *Lls1*

Transposon tagging with *Mutator* (*Mu*) was used to clone *Lls1*. Two different tagging strategies were used that resulted in the isolation of six mutants (Johal et al., 1994). Briefly, the directed mutagenesis approach, which involved a cross between *Mu*-containing *Lls1*/*Lls1* females and a mutant tester (*lls1-ref*/*lls1-ref*, containing the reference mutant allele isolated by Ullstrup and Troyer in 1967), yielded two mutants, *lls1-1* and *lls1-2*, from a screen of about 30,000 plants. In addition, *lls1* mutant sectors of varying sizes were also observed on a number of plants (Figure 1C). From a random mutagenesis approach, in which more than 24,000 *Mu*-active M2 families were screened for the *lls1* mutant phenotype, four more germinal mutants, *lls1-3*, *lls1-4*, *lls1-5*, and *lls1-6*, were derived. Initial propagation of all six of these mutants has been described (Johal et al., 1994), except that the mutant allele–carrying progeny of both *lls1-1* and *lls1-2* needed to be first distinguished with *lls1*-linked RFLP markers before further propagation (these *Mu*-generated mutant alleles were in repulsion with the *lls1-ref* allele in the original mutants).

A DNA gel blot analysis was used to search for cosegregating *Mu* transposons responsible for the mutations (Johal and Briggs, 1992; Walbot, 1992). Cross-hybridization of each of the nine *Mu* elements was examined with DNA samples isolated from each of the mutant families segregating 1:1 for plants containing and lacking the mutant allele. The progeny that were used for cosegregation analyses were produced by pollinating a wild-type (WT) plant, heterozygous for a given mutant allele, with pollen from a plant that was homozygous for the *lls1-ref* allele. A *Mu8* element, contained in a 3 kb EcoRI restriction fragment, showed complete linkage with the *lls1-5* allele among a sample of 66 DNAs (Figure 2A). This 3 kb fragment was cloned, and DNA flanking the *Mu8* insertion was PCR-amplified and subcloned (Figure 2B).

### Cloning Confirmation

Due to the low rate of *Mu* germinal excision (Walbot, 1992), which prevented us from verifying the cloning of *Lls1* by mutant reversion, our approach relied on the premise that if our clone originated from *lls1*, it should detect structural rearrangements in the other mutant alleles. Since most of our mutants were likely to be caused by *Mu* insertions, a PCR-based method was devised to detect the presence of this transposon in all *lls1* mutants. The RF5 (right flank of *Mu* insertion in *lls1-5*) fragment was sequenced, and an RF5-specific primer,

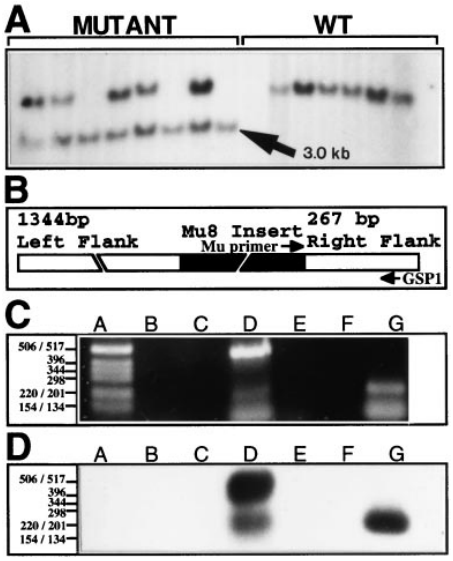


Figure 2. Cloning and Confirmation of the *lls1* gene

(A) Cosegregation of a 3 kb *Mu8*-hybridizing (arrow) restriction fragment with the *lls1-5* mutant allele. DNA samples (10 μg) from eight mutant (*lls1-5*/*lls1-ref*; lanes 1–8) and seven WT siblings (*Lls1*/*lls1-ref*; lanes 9–15) were digested with EcoRI.

(B) Diagram of the 3.0 kb EcoRI clone, showing the position of *Mu8* in relation to the left (1344 bp) and the right (267 bp, RF5) flanks. Locations and directions of GSP1 (an RF5-specific primer) and *Mu*-TIR primers are shown.

(C) Gel visualization of PCR products amplified from the DNA of an *lls1-2*/*lls1-ref* mutant and an *Lls1*/*lls1-ref* WT sib using a combination of GSP1 and *Mu*-TIR primers. Lane A (1 kb marker; sizes of individual marker fragments are shown on the left). Template DNAs were from the mutant (lanes B–D) and WT sibs (lanes E–G). Amplification reactions were carried out in the presence of GSP1 primer alone (lanes B and E), *Mu*-TIR primer alone (lanes C and F), and with a combination of GSP1 and *Mu*-TIR primers (lanes D and G).

(D) Cross-hybridization of the PCR products in (C) with a probe made from RF5 (B).

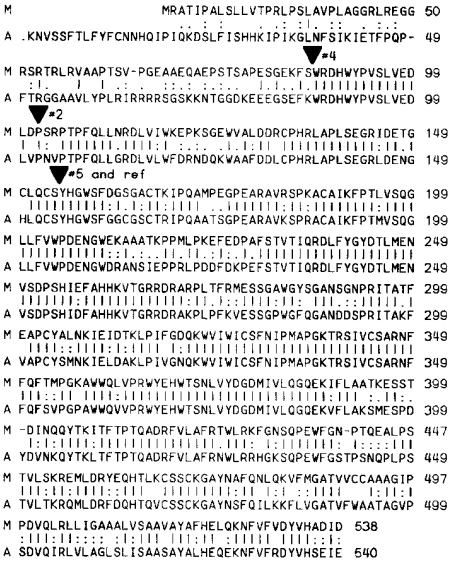


Figure 3. Comparison of the Maize Predicted LLS1 Protein with a Putative Homolog from Arabidopsis

Alignment of the maize predicted LLS1 protein (521 amino acids) with a continuous ORF predicted from a contig of four overlapping Arabidopsis EST clones. Proteins were aligned using a Lipman-Pearson algorithm of the ALIGN program of the DNASTAR DNA software package (K-tuple, 2; gap penalty, 4; gap length penalty, 12). *Mu* insertional sites of four mutants, #2 (*lls1-2*), #4 (*lls1-4*), #5 (*lls1-5*) and ref (*lls1-ref*), are shown as closed triangles.

GSP1 (Figure 2B), was designed and used in combination with a *Mu*-TIR (terminal inverted repeat) primer in a PCR reaction with template DNA isolated from each of the other five mutants. Two products were amplified from *lls1-2*-bearing plants (Figure 2C). The longer product (434 bp) was amplified only from the mutant allele in the segregating family, while the smaller product was found in all the progeny. Both PCR products cross-hybridized to the RF5 probe, indicating that they were amplified from this region of the chromosome (Figure 2D). Sequence analysis showed that *Mu* had inserted at 246 bp 5′ of the *Mu8* -insertion site of *lls1-5* in the *lls1-2*-specific fragment. A similar result was obtained with the *lls1-4* allele, revealing a *Mu* insertion 292 bp 5′ of the *lls1-5* site (data not shown).

The smaller product (189 bp), which amplified from all the progeny of all five mutants, was the unexpected result of a *Mu* insertion in the *lls1-ref* allele. The *Mu* element in *lls1-ref* is inserted at exactly the same location as the *Mu8* element in *lls1-5*. However, they seem to be the result of independent transpositional events, because an unusually large duplication adjoins the *Mu8* insertion in the *lls1-5* allele but is absent in the *lls1-ref* allele (data not shown).

### The *Lls1* Gene Encodes a Novel Plant Protein

To ascertain the nature of the *Lls1* gene product, RF5 was used as a probe to isolate 3 cDNAs from a maize seedling library. The sequence of the longest cDNA (pJG200, 1.678 kb) was determined, which in turn allowed us to recover a longer cDNA clone (1.85 kb) from the maize EST collection at Pioneer Hi-Bred International, Inc. The sequence of the two *lls1* cDNAs was compiled into a single span of 1,855 nucleotides. A genomic clone was isolated, from which a 7.1 kb SacI fragment containing the entire *lls1* gene was subcloned (pJG201) and sequenced.

Primer extension analysis predicted a transcript of 2119 bp, and it matched perfectly in size with the *lls1* transcript detected on Northern blots of leaf RNA (data not shown). Although our longest cDNA is about 250 bp shorter than the predicted transcript, the fact that it contains the first in-frame ATG codon suggests that our cDNA contains the entire coding region of 521 amino acids of *Lls1* (Figure 3). The expected molecular mass of the predicted LLS1 is 58 kDa, and it appears to be hydrophilic, with a pI of 7.5. A search of all available databases revealed that, except for four expressed sequence tags (ESTs) from the Arabidopsis thaliana database with unknown function (discussed later), the *lls1* gene encodes a novel polypeptide.

### *lls1* Is Conserved between Monocots and Dicots

A garden blot of DNAs derived from both monocot and dicot plants was used to address whether *lls1* is unique to maize or is also present in other plant species (data

not shown). Cross-hybridizing sequences were detected among all monocots tested, suggesting that the function of *lls1* may extend beyond maize. Although no discrete hybridization signal could be detected with any of the dicot DNAs during this experiment, a gene was found in the Arabidopsis EST database that exhibited 70% sequence identity (nucleotide level) with the maize *lls1* gene. This EST and three other overlapping ESTs were obtained from the ABRC (Columbus, OH), and their sequence allowed us to organize them into a span of 1977 bp. An ORF of 540 amino acids is predicted from this span; it exhibits 71.6% amino acid identity with LLS1 over 473 residues (Figure 3), including termination at the same codon. For the first 65 amino acids at the amino terminus, there is little homology between the maize and Arabidopsis genes. No initiator methionine could be detected in the Arabidopsis gene.

### The Predicted LLS1 Protein Contains Two Structural Motifs Highly Conserved in Bacterial Phenolic Dioxygenases

While no definite function could be ascribed to *lls1* from homology searches, analysis of the predicted amino acid sequence of the *lls1* gene product has revealed two conserved motifs, a consensus sequence (Cys-X-His-$X_{16-17}$-Cis-$X_2$-His) for coordinating the Reiske-type [2Fe–2S] cluster (Mason and Cammock, 1992) and a conserved mononuclear nonheme Fe-binding site (Glu-$X_{3-4}$-Asp-$X_2$-His-$X_{4-5}$-His) (Jiang et al., 1996), which are present in the $\alpha$-subunit of all aromatic ring-hydroxylating (ARH) dioxygenases involved in the degradation of phenolic hydrocarbons (Figure 4A). In addition, the spacing ($\sim$90 amino acids) between these motifs, which has recently been shown to be conserved in all ARH dioxygenases, is precisely maintained in LLS1, adding further evidence that LLS1 may encode a dioxygenase function. The ARH dioxygenases consist of 2 or 3 soluble proteins that interact to form an electron transport chain that transfers electrons from NADH via flavin and iron–sulfur (2Fe–2S) redox centers to a terminal dioxygenase. The latter, which is also a multimeric enzyme consisting of either $\alpha$ homomers or $\alpha$ and $\beta$ heteromers, catalyzes the incorporation of two hydroxyl groups on the aromatic ring at the expense of dioxygen and NAD(P)H.

The consensus sequence of both the Rieske- and iron-binding motifs, as well as the spacing between them, are precisely conserved in a hypothetical protein (translated from an ORF) from Synechocystis sp. PCC6803 (Figure 4B), which, in addition, exhibits 66% amino acid identity to LLS1 among a stretch of more than 100 amino acids. Additionally, the Rieske center–binding site has also been detected in the partial sequence of two seemingly related ESTs of unknown function, one each from rice and Arabidopsis (Figure 4A).

## Discussion

It is becoming increasingly apparent that lesion mimic mutations in plants probably represent malfunctions of genetic controls and mechanisms of PCD, which in plants, as in animals, play a central role in both development and maintenance (Gahan, 1981; Johal et al., 1995; Jones and Dangl, 1996). The prevalence of lesion mimics in maize and Arabidopsis suggests that, as in animals, multiple pathways may exist that implement this cell death program in plants (Johal et al., 1995; Dangl et al., 1996). However, in contrast to animal systems, where a clear understanding of the biology of this process has emerged (Stellar, 1995; Jacobson, et al., 1997; Nagata, 1997), the mechanistic nature of plant cell death is largely unknown. The isolation of *lls1*, recessive mutations of which clearly suggest that it plays a key role in limiting the spread of cell death, therefore provides a molecular tool to explore cell death in plants.

The timed and mainly leaf-specific expression of the *lls1* mutant phenotype argues that LLS1 may not be required globally in the plant. Additional genes, structurally related or unrelated to LLS1, may suppress cell death in tissues other than the mature leaf. The cell-autonomous nature of *lls1* mutations implies that LLS1 is a component of a cell death pathway that functions intracellularly. Whether LLS1 plays any role in the HR cell death pathway is unclear at present, although *lls1* lesions can be readily triggered by mechanical wounding (Close et. al., 1995). In fact, *lls1* lesions can be set off by any kind of cell-damaging stimulus, including the HR, genetic lesions, and physiological stresses (J. G. and G. S. J., unpublished data), suggesting that the cell death pathway that is suppressed by LLS1 may lie downstream of most, if not all, cell death pathways in plants.

The *lls1* gene was cloned via transposon tagging with *Mu*. That the correct gene has been cloned is apparent from having four different *Mu* insertional events within a stretch of 300 bp in four independent mutant alleles of *lls1*. Since in a genome of $\sim 3 \times 10^9$ bp, the probability of this happening by chance is extremely small (1 in $10^{27}$), independent insertions are considered a proof for the correct cloning of a gene (Johal and Briggs, 1992; Walbot, 1992). As usual, *Mu* insertions in all *lls1* mutants characterized are in the 5′ end of the coding region (Bennetzen et al., 1993). To our knowledge, the *Mu* insertion in the *lls1-ref* allele marks the first indication of *Mu*'s transpositional activity prior to its discovery in 1978 (Robertson, 1978).

### *lls1* Encodes a Novel Function Conserved in Plants

Since no homologs of the *lls1* gene could be detected in any of the nonplant databases, including yeast, it suggests that the cell survival function encoded by *lls1* either never evolved in animals or has diverged to the point that it is no longer detectable at the sequence level. In contrast, the *lls1* gene appears to be highly conserved across the evolutionary divide between monocots and dicots. An Arabidopsis complementary DNA clone of unknown function that exhibits 72% amino acid identity to the maize *lls1* gene was found in an EST database. In sorghum, a recessive mutation, called *drop dead* (*dd1*), which mimics the maize *lls1* mutation in every respect including syntenic organization, has been identified, indicating that it encodes the same function as the maize *lls1* gene (J. Theuri and J. S. G., unpublished data). The presence of an *lls1*-homologous ORF in the genome of Synechocystis, a photosynthetic cyanobacterium, raises the possibility that it may be the ancestor

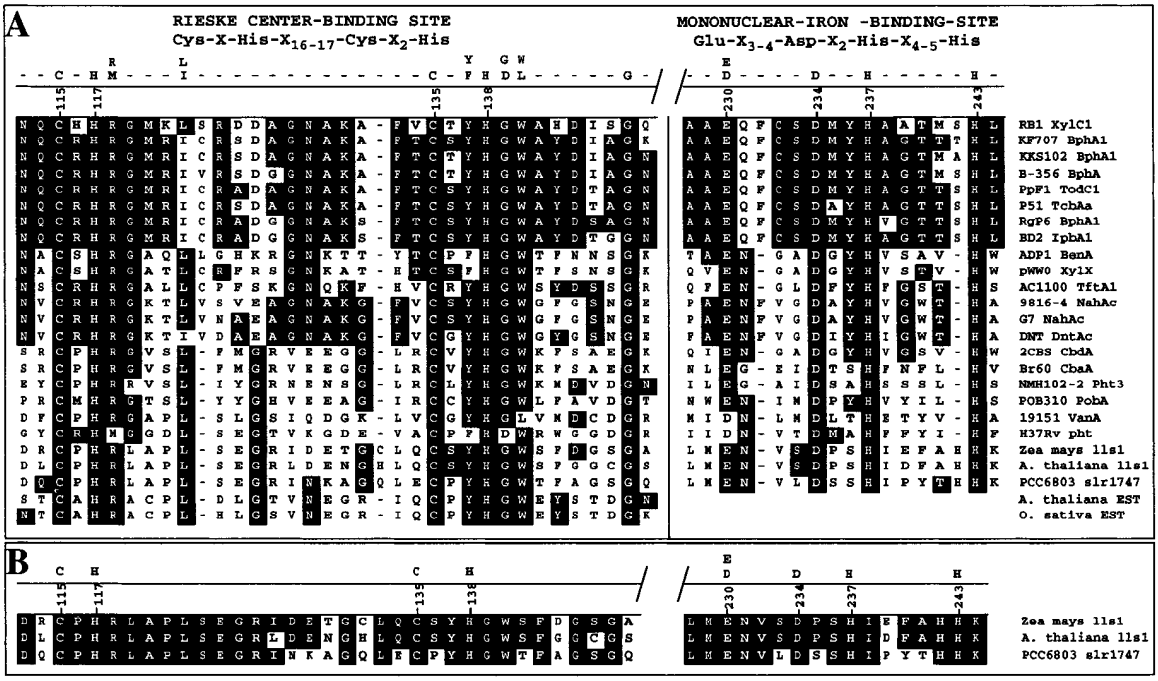


Figure 4. Identification and the Nature of Consensus Motifs in the Predicted LLS1 Protein

(A) Amino acid sequence alignments of the Rieske [2Fe–2S]- and the mononuclear iron–binding regions of the $\alpha$ subunits of a number of bacterial ARH dioxygenases with the predicted proteins of the maize *lls1*, its homologs from Arabidopsis and Synechocystis strain PCC6803 (slr1747), and the partial ORFs of two additional ESTs (nonhomologous to *lls1*) from Arabidopsis and rice. Except for the following four, information on all other bacterial ARH dioxygenases is present in Jiang et al. (1996): RB1 *XylC1*, putative biphenyl dioxygenase from Cycloclasticus oligotrophus; B-356 *BphA*, biphenyl dioxygenase from Comomonas testeroni strain B356; BD2 *ipbA1*, isopropylbenzene 2,3-dioxygenase from Rhodococcus erythropolis strain BD2; H37Rv *pht*, putative phthalate dioxygenase from Mycobacterium tubercolosis strain H37Rv. Conserved motifs are displayed above the alignments. Residues conserved in all proteins are shaded black. Residue positions reflect those of the predicted maize LLS1 protein, residue 1 being the first methionine.

(B) Alignment of the maize predicted LLS1 in the region overlapping both consensus motifs with the corresponding regions of *lls1* homologs from Arabidopsis and Synechosystis.

of the plant LLS1 function. This possibility, along with the fact that *lls1* lesion progression is dependent on light harvested during photosynthesis (Close et al., 1995), suggests that LLS1 may have evolved to protect autotrophic organisms from cell-damaging stresses that build up during photosynthesis (Reinbothe et al., 1996).

### LLS1 May Function as a Dioxygenase

Although LLS1 is a novel protein, it does have two consensus motifs, a Rieske-type iron–sulfur binding site and a mononuclear iron-binding site, suggesting that it may function as an aromatic ring-hydroxylating (ARH) dioxygenase. Dioxygenases are common among soil-inhabiting bacteria, where they are involved in the aerobic degradation of aromatic hydrocarbons. The first step of this degradation pathway is often mediated by ARH dioxygenases, which catalyze the incorporation of both atoms of molecular oxygen in their substrates to form a *cis*-dihydrodiol. This reaction is then followed by a dehydrogenase reaction before the aromatic ring is opened by a ring-cleaving dioxygenase (Mason and Cammock, 1992; Jiang et al., 1996). If confirmed, *lls1* would be the first ARH dioxygenase gene described from plants. Apparently, it is not the only one in plants, as another EST with the Rieske-type iron–sulfur binding domain is present in both the Arabidopsis and rice databases.

The probable dioxygenase nature of the *lls1* gene suggests that its target in the plant, the mediator of cell death, may be a phenol. Ubiquitously present, phenolic compounds are known to play diverse regulatory and functional roles, including roles in disease resistance (Dixon and Paiva, 1995). The typical browning reaction that is associated with HR and with cut fruits and vegetables is attributed to oxidation products of phenolics (Appel, 1993). While such oxidative reactions serve to restrict pests and pathogens, they are also detrimental to plant cells. Although normally inert, most phenolics become highly reactive under oxidative conditions, such as those caused by photooxidative, respiratory, or other biotic and abiotic stresses, and readily donate their electrons to oxygen, generating ROS and phenolic free radicals (semiquinones) as a consequence (Appel, 1993). If not countered, this situation can lead to irreversible damage to cell organelles and, eventually, death. The requirement of *lls1* lesions for both cell damage and light energy (Close et al., 1995; J. G. et al., unpublished data) is consistent with a phenolic compound mediating cell death in *lls1* plants. The observation that the level of a phenolic compound of uncertain identity is elevated

in *lls1* leaves lends support to this contention (Obanni et al., 1994).

One candidate that may fit well in this role is salicylic acid (SA). SA, which exhibits a 10- to 50-fold increase during the HR, is also triggered in response to oxidative stresses associated with ozone or UV exposure (Hammond-Kosack and Jones, 1996; Ryals et al., 1996). In addition, SA is known to cause $H_2O_2$ buildup (Chen et al., 1993), transmute into a cell-damaging free radical under oxidizing conditions (Durner and Klessig, 1996), and be involved in cell death associated with a number of Arabidopsis *lsd* mutants (Dangl et al., 1996; Weyman et al., 1996). Although these characteristics of SA tempt us to speculate that it may be a mediator of cell death in *lls1* mutants, the possibility nevertheless remains that a novel compound or mechanism is responsible for *lls1*-associated cell death.

The predicted association of LLS1 with an iron–sulfur cluster suggests that it may also participate in oxidation–reduction reactions. Proteins that use iron–sulfur clusters as prosthetic groups often function as biosensors of oxidants and iron (Roualt and Klausner, 1996). It is conceivable, therefore, that LLS1 may also serve as a kind of rheostat such as that proposed for LSD1 in regulating cell death in plants (Jabs et al., 1996).

## Experimental Procedures

### Plant Material

The original *lls1* mutant, containing the reference allele, was obtained from the Maize Genetics Coop., University of Illinois, Urbana/Champaign. Stocks containing active *Mu* transposons were obtained from Dr. D. Robertson, Iowa State University. The six transposon-tagged mutant alleles, *lls1-1* through *lls1-6*, were previously designated as *lls**-29215, *lls**-42230, *lls**-1127, *lls**-1424, *lls**-3744, and *lls**-4911, respectively (Johal et al., 1994).

### Cosegregation Analysis and Cloning

DNAs were extracted either by a urea- (Dellaporta et al., 1983) or CTAB-based (Hulbert and Bennetzen, 1991) protocol. For cosegregation analysis, pooled (15–30) DNAs from either mutant or wild-type siblings of each mutant were digested individually with seven restriction enzymes. Southern blot analysis was performed essentially as described by Gardiner et al. (1993), and the blots were hybridized with each of the 9 *Mu* elements. Following identification of the *Mu8*-hybridizing, 3.0 kb EcoRI restriction fragment as a possible *lls1* candidate from *lls1-5*, its linkage with the mutant allele was examined with 66 plants.

The 3.0 kb EcoRI restriction fragment containing the *Mu8* element was cloned from an *lls1-5*/*lls1-ref* plant in the λ Zap Express vector (Stratagene) and excised as a phagemid. The DNA fragments flanking *Mu8* insertion in this clone were amplified using a *Mu*-TIR primer (5′ CGCCAACGCCTCCATTTCGTCGAATCC 3′) and either the forward or the reverse vector-specific primers. The amplified product from the right flank (RF5) was subcloned in the TA Cloning vector (Invitrogen). The primer sequences for confirmation analysis were 5′ TGGGGAACTTGATCGCGCACGCCTTCGG 3′ (GSP1) and the *Mu*-TIR primer described above. The PCR conditions were: 94°C for 5 min; then cycled 40 times for 30 sec at 94°C, 1 min and 30 sec at 62°C, and 1 min at 72°C; and, finally, 5 min at 72°C. The amplified products were cloned in the TA vector for sequencing. To isolate cDNA clones, a seedling leaf cDNA library from the inbred Pa405, constructed in the λ Zap Express (Stratagene) vector, was probed with RF5. To isolate an *lls1* genomic clone, a B73 partial Sau3AI library in λ DashII (Stratagene) was screened with RF5 and a single positive clone was recovered, followed by subcloning of a 7.1 kb SacI fragment in pBluescript KS(+) (Stratagene).

### Primer Extension and RNA Blot Analysis

An oligonucleotide, GSP22 5′ GTGCTCGGCTCCGCCTGCTCCGCC GCTTCCCCTGG 3′, complementary to nucleotides 139–173, downstream of the predicted first in-frame ATG of the coding strand, was synthesized and end-labeled with $^{32}P$. Primer extension analysis was performed according to McKnight et al. (1981), except for the following modifications. Total RNA (40 μg) from immature tassels of B73 and 0.2 pmol of labeled oligonucleotide was annealed at either 33°C, 37°C, 45°C, or 55°C for 4 hr. Following primer extension, samples were incubated at 37°C for 30 min with 2 μl of 0.5 M EDTA and 1 μl of mixed RNAases (0.5 mg/ml RNAase A and 10,000 units/ml RNAase T1; Ambion) to degrade free RNA. Blot analysis of mRNA from mature maize leaves was carried out as described previously (Johal and Briggs, 1992) using the entire cDNA clone as a probe.

### DNA Sequencing and Analysis

DNA was sequenced using a SequiTherm Cycle Sequencing Kit (Epicentre, Madison WI) according to the manufacturer's instructions. The sequences of the *lls1* cDNA, the 7.1 kb genomic fragment, and the Arabidopsis homolog have been deposited in GenBank. Local sequence comparisons were performed using ALIGN and MEGALIGN programs of the DNASTAR software package (DNASTAR Inc., Madison, WI). Algorithms employed were the neighborhood search algorithm BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) or BEAUTY (Worley et al., 1995). Searches of the GenBank databases were performed using the National Center for Biotechnology Information's BLAST WWW Server.

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**GenBank Accession Numbers**

Accession numbers for the sequences reported in this paper are as follows: *Lls1* cDNA, U77345; *Lls1* gene, U77346; Arabidopsis *Lls1* U77347. Accession numbers for *Lls1*-related expressed sequence tags or gene sequences from various organisms are as follows: Arabidopsis, H36617, N37395, R30609, T45298, T22255; Rice, D46313; Synechocystis, D60909; Cycloclasticus oligotrophus, U51165; Comomonas testeroni, U47637; Rhodococcus erythropolis, U24277; Mycobacterium tuberculosis, Z82098.